Establishing a model of primary bovine hepatocytes with responsive growth hormone receptor expression

S. Witte,¹ Y. Brockelmann,¹ J.-D. Haeger,² and M. Schmicke^{1*†}

¹Clinic for Cattle, Endocrinology Laboratory, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, 30173, Germany

²Institute for Anatomy, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, 30173, Germany

ABSTRACT

The liver becomes resistant to growth hormone before parturition in dairy cows (uncoupling of the somatotrophic axis). However, the mechanism of growth hormone insensitivity has not been fully described. The aim of the present study was to improve a previous model of adult bovine hepatocytes in a sandwich culture system to ensure growth hormone receptor (GHR) expression. First, we modified the protocol for hepatocyte retrieval and tested the effect of short (18 min) and long (up to 30 min) warm ischemia on hepatocyte viability. Second, we used medium additives that affect GHR expression in vivo—insulin (INS), dexamethasone (DEX), both (INS+DEX), or no hormone additives (CTRL)—to ensure the functionality of hepatocytes, as measured by lactate dehydrogenase activity and urea concentration in the medium. We also used reverse transcriptase PCR of hepatocytes to evaluate expression of albumin (*ALB*), hepatocyte nuclear factor 4 α (*HNF4A*), nuclear factor- κ B-inhibitor α (*NFKBIA*), cytosolic phosphoenolpyruvate carboxykinase (*PCK1*), and vimentin (*VIM*) mRNA. Moreover, we analyzed the expression of GHRtot (*GHR*), *GHR1A*, insulin-like growth factor-1 (*IGF1*), and IGF binding protein-2 (*IGFBP2*) in response to exposure to media with the different compositions. Modification of the protocol (changes in rinsing and perfusion times, buffer composition, and the volume and standardization of collagenase) led to increased cell counts and cell viability. Short warm ischemia with the modified protocol significantly increased cell count ($4.7 \times 10^7 \pm 1.9 \times 10^7$ vs. $3.5 \times 10^6 \pm 1.5 \times 10^6$ vital cells/g of liver) and viability (79.1 ± 8.4 vs. $37.1 \pm 8.9\%$). Therefore, we gathered hepatocytes from the liver after short warm ischemia

with the modified protocol. For these hepatocytes, lactate dehydrogenase activity was lower in media with INS and with DEX than in media with INS+DEX or CTRL; urea concentrations were highest at d 4 for INS+DEX. As well, *HNF4A* and *ALB* were more highly expressed in hepatocytes cultured with INS and INS+DEX than in those cultured with DEX or CTRL, and the substitution of DEX suppressed *VIM* and *NFKBIA* expression but increased *PCK1* expression. The expression of *GHR*, *GHR1A*, and *IGF1* was suppressed by dexamethasone (DEX and INS+DEX), whereas INS distinctly increased *GHR*, *GHR1A*, and *IGF1* mRNA expression. Hepatocytes in a sandwich culture showed influenceable *GHR* expression; this study provides a model that can be used in studies examining factors that influence the expression and signal transduction of GHR in dairy cows.

Key words: growth hormone, insulin-like growth factor, hepatocytes, sandwich culture

INTRODUCTION

The liver is one of the most important organs in the body and serves a variety of major functions, including metabolic and immunological ones. It plays a key role in carbohydrate, protein, and fat metabolism and has been considered in many studies in high-yield dairy cows. To examine the function of the liver in detail, well-described in vitro models are necessary. The present study focuses on establishing a model to examine hepatic growth hormone receptor (GHR) regulation, because in high-yield dairy cows, the liver becomes resistant to growth hormone before parturition (Kobayashi et al., 1999), called the uncoupling of the somatotrophic axis (Lucy et al., 2001). However, the mechanism behind growth hormone resistance has not yet been fully elucidated. The expression and signal transduction of GHR can be regulated at several levels, for example by modifying receptor expression, surface availability and GHR signaling, which involves at least 3 major pathways: signal transducer and activator of transcrip-

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*Corresponding author: marion.schmicke@Landw.uni-halle.de

†Martin-Luther Universität Halle-Wittenberg, 06020 Halle, Germany.

tion proteins (STAT); mitogen-activated protein kinase (MAPK); and phosphoinositide 3-kinase (PI3)/Akt. Various immune factors and hormones such as insulin, thyroid hormones, cortisol, and sex hormones can modulate GHR through some of these mechanisms (Flores-Morales et al., 2006). Dexamethasone downregulates GHR expression and decreases peripheral IGF-1 in cows after 5 d without affecting IGF binding proteins (IGFBP; Bennett et al., 1996; Maciel et al., 2001). In dairy cows, low insulin concentrations are thought to be responsible for the decreased expression of GHR, and the infusion of cows with supraphysiological concentrations of insulin increases GHR expression in the liver (Butler et al., 2003). Other studies indicate that insulin might be only one of the factors that affect periparturient GHR expression (Leung et al., 2003; Winkelman et al., 2008). Cows with very different IGF-1 levels but comparable GHR expression also show comparable insulin levels (Piechotta et al., 2013, 2014). Therefore, not only is GHR expression likely impaired, but also GHR signal transduction. Winkelman et al. (2008) found increased *SOCS2* expression around calving, but which factor induces *SOCS2* expression remains unclear. Therefore, we established a cell culture model for detailed study (Ehrhardt and Schmicke, 2016). Ehrhardt and Schmicke (2016) had 2 major limitations when using a model of primary bovine hepatocytes from abattoir-derived liver. First, the gathered cell count was low, and second, GHR expression was impaired. The aim of the present study was to evaluate the effect of factors during hepatocyte gathering and the effect of insulin and dexamethasone on cell count and GHR expression in adult primary bovine hepatocytes to modify isolation and culture conditions to ensure GHR expression.

MATERIALS AND METHODS

Experimental Design

To ensure GHR expression in a primary hepatocyte cell culture, we hypothesized that modifying the 2-step collagenase perfusion and culturing conditions led to basal and reactive GHR expression. Therefore, we designed 2 experiments. In experiment 1, we modified a previously published protocol for gathering primary bovine hepatocytes from abattoir-derived liver and tested whether cell count and cell viability (ratio of live to dead cells) could be improved compared with the original protocol. We also evaluated the influence of the duration of warm ischemia on cell yield and viability in primary bovine hepatocytes depending on the protocol (original versus modified). In experiment 2, we cultured

the cells in a sandwich culture and tested the influence of insulin and dexamethasone on GHR expression.

Experiment 1: Isolation of Primary Hepatocytes and Influence of Warm Ischemia

Based on the previously published protocol for isolation of bovine hepatocytes (Ehrhardt and Schmicke, 2016), we established the following modified protocol that differed primarily in terms of buffer composition, increased buffer volume for rinsing, and temperature of rewarming (37°C) and stop solution (4°C). Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). In brief, the following steps were modified. The liver was rinsed via vessels at the surface of the 30- to 90-g part of the lobus caudatus with 250 mL of EGTA buffer (4°C) and then with 200 mL of EGTA-free buffer (4°C, Table 1). The liver was then transported to the cell culture laboratory in a glass jar with 250 mL of EGTA-free buffer on ice. The time between the rinsing of the liver part and the start of the perfusion was 30 to 90 min. In the cell culture laboratory, the liver was rewarmed with 150 mL of EGTA-free buffer (37°C). Then, a 2-step collagenase perfusion was performed using EGTA buffer (39°C) for 5 min, CaCl₂ buffer (39°C) for 5 min, and 1 mg of collagenase P per gram of liver in CaCl₂ buffer for 5 to 7 min. Afterward, the liver was placed on ice again, and cells were flushed out with stop solution (4°C, Williams' Medium E with 20% FBS; PAN-Biotech, Aidenbach, Germany). The cells were then centrifuged (60 × *g*, 3 min, 4°C), and cell count and cell viability were determined as described by Ehrhardt and Schmicke (2016). Vital hepatocytes were purified using 75% PBS and 25% Easycoll (density 1.124 g/mL; Merck, Darmstadt, Germany) and centrifuged for 8 min at 1,000 × *g* and 4°C. After purification, we measured cell count and cell viability again.

To validate the influence of the liver material on cell viability and cell count, we compared livers from 2 different sources. First, we used livers with a long warm ischemia time (**LWI**; *n* = 12, 28–30 min from slaughtering to cold buffer) from a commercial slaughterhouse, comparable to those used in Ehrhardt and Schmicke (2016). Second, livers from primiparous cows 30 d after calving were available from the "Chron mast project" (experiment approved by the Lower Saxony Federal State Office for Consumer Protection and Food Safety under the reference number 33.12-42502-04-15/2024; *n* = 12, 18 min from slaughtering to cold buffer, short warm ischemia time, **SWI**). These 24 livers from the 2 sources were perfused using the original protocol (Eh-

Table 1. Composition of buffers used in the 2-step collagenase perfusion of the caudate liver lobe obtained after death from an adult cow: modified protocol (present study) versus the original protocol (Ehrhardt and Schmicke, 2016)

Modified isolation protocol			Original isolation protocol		
Buffer	Reagent	Concentration	Buffer	Reagent	Concentration
EGTA buffer	Amino acids	0.01%	Perfusion buffer I	Amino acids	0.01%
	Dexamethasone	100 nM		Dexamethasone	100 nM
	EGTA	1 mM		EGTA	1 mM
	Glucose	20 mM		Glucose	5 mM
	HEPES	20 mM		HEPES	9.2 mM
	ITS supplement	20/11/0.01 µg/µL		Bovine insulin	35 nM
	KCl	2.4 mM		KCl	0.5 mM
	KH ₂ PO ₄	1.2 mM			
	NaCl	142 mM		NaCl	142 mM
	Na-pyruvate	10 µM		Na-pyruvate	10 µM
CaCl ₂ buffer	Amino acids	0.01%	Perfusion buffer II	CaCl ₂ ·H ₂ O	5.4 mM
	CaCl ₂ ·H ₂ O	5.4 mM			
	Dexamethasone	100 nM			
	Glucose	20 mM		HEPES	9.2 mM
	HEPES	20 mM		KCl	6.7 mM
	ITS supplement ¹	20/11/0.01 µg/µL			
	KCl	2.4 mM		NaCl	68 mM
	KH ₂ PO ₄	1.2 mM			
	NaCl	142 mM			
	Na-pyruvate	10 µM			
Collagenase		1 mg of collagenase P/g of liver in 100 mL of CaCl ₂ buffer	Collagenase		90 mg of collagenase P in 100 mL of perfusion buffer II

¹ITS = insulin-transferrin-selenium.

rhardt and Schmicke, 2016; n = 6 SWI, n = 6 LWI) or the modified protocol (n = 6 SWI, n = 6 LWI).

Experiment 2: Sandwich Culture of Primary Hepatocytes and Effect of Insulin and Dexamethasone on GHR Expression

After hepatocytes were isolated using the modified protocol, they were seeded on collagen-coated 12-well plates (1.37×10^5 vital cells/cm²) in Williams' Medium E with 10% fetal bovine serum. After 3 h, the cells were washed with 10% PBS, and a second collagen layer was added to prepare a sandwich culture. Hormone-free Williams' Medium E was replaced after the second collagen layer was solidified. Cells were cultured in a humidified atmosphere at 37°C and 5% CO₂. The medium was changed 18 h after separation to a hormone-defined medium that was equivalent to Williams' Medium E with dexamethasone (DEX), insulin (INS), both (DEX+INS), or neither (CTRL).

The bovine hepatocytes were cultured in standard medium for liver cells, which is hormone-defined Williams' Medium E containing penicillin/streptomycin (100 U/mL, 0.1 mg/mL), gentamicin (50 µg/mL), amphotericin B (2.5 µg/mL), and L-glutamine (4 mM). In Ehrhardt and Schmicke (2016), INS (100 nM) and DEX (100 nM) were added to the medium. Both

hormones influence GHR expression in vitro and in vivo. Therefore, we isolated bovine hepatocytes with a SWI and the modified protocol with either INS, DEX, INS+DEX or CTRL. In the cell culture medium, we measured lactate dehydrogenase (LDH) activity and urea as parameters indicating liver cell viability, and in cell samples, we determined genes of interest (Table 2).

Laboratory Analyses

Live/Dead Stain. To evaluate the viability and morphology of the primary bovine hepatocytes at d 1 and 4 after perfusion, the cells were stained using a LIVE/DEAD Viability/Cytotoxicity Kit (ThermoFisher Scientific, Waltham, MA) and following the manufacturers' instructions (<https://www.thermofisher.com/order/catalog/product/L3224>). We observed the results using an inverse fluorescence microscope (Axiovert 200M; Zeiss, Oberkochen, Germany). From 6 wells of INS, DEX, INS + DEX, and without any hormonal additive (CTRL), micrographs were taken and cells were counted and the percentage of dead/live cells was calculated in one visual field.

Urea and Lactate Dehydrogenase Measurement in Cell Culture Medium. The urea concentration was measured using the Urea Assay Kit ab83362 (Abcam, Cambridge, UK), and LDH activity using a

Lactate Dehydrogenase Activity Assay Kit (Sigma-Aldrich). Both assays were performed according to the manufacturers' instructions (<https://www.sigmaaldrich.com/catalog/product/sigma/mak066?lang=de®ion=DE>). The intra-assay coefficients of variation were 6.1% for urea and 5.2% for LDH.

mRNA Isolation and Quantitative Reverse Transcription PCR. We determined the expression of *HNF4A*, *NFKBIA*, *ALB*, *PCK1*, *VIM*, *GHR*, *GHR1A*, *IGF1*, and *IGFBP2* mRNA in the cell samples after 6 and 72 h of incubation using quantitative reverse transcriptase PCR. Therefore, total RNA was extracted using Trizol Reagent (Sigma-Aldrich) according to the original protocol (Ehrhardt and Schmicke, 2016). To assess the quality and quantity of the extracted RNA, we determined relative integrity using an RNA 6000 Nano Assay Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). To transcribe RNA into cDNA, we used a CFX96 C1000 Touch thermocycler (Real-Time PCR detection system, BioRad, Munich, Germany) as previously described (Piechotta et al., 2013). For the genes of interest, the PCR reaction mix contained 1.25 ng/ μ L cDNA, 4 μ L of HOT FIREPol EvaGreen qPCR Supermix (5 $\times$; Soles BioDyne, Tartu, Estonia), and 0.2 μ M each primer (Eurofins MWG Operon, Ebersberg, Germany). The primers were either previously described or constructed based on information in a public database (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>). The primer sequences and accession numbers are shown in Table 2. The PCR cycle was programmed as fol-

lows: activation of the DNA polymerase at 95°C for 720 s, followed by 40 cycles of RNA denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. We used HOT FIREPol EvaGreen to visualize the fragments. A melting curve was generated to verify PCR fragments. The PCR was initiated at 55°C, increasing to a final temperature of 95°C, with a temperature increase of 0.5°C every 5 s. Finally, we determined the relative abundance of genes of interest relative to a calibrator (cells were collected immediately after the purification step with Easycoll and directly frozen at -80°C until analyses) and calculated it using the $\Delta\Delta\text{Ct}$ method with respect to the different efficiencies of the primers (Pfaffl, 2004). We used *GAPDH* and peptidylprolyl-isomerase A (*PPIA*) as reference genes. The mRNA expression of both reference genes was tested for effects of time or treatment and it was not affected and therefore suitable as reference genes.

Statistical Analyses

The statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). First, we tested data for normal distribution using the Shapiro-Wilk test: all data were normally distributed. In experiment 1, to test the main effect of the isolation protocol (original versus modified), warm ischemia, and the interaction between isolation protocol and warm ischemia, we performed a 2-way ANOVA using a post hoc Sidak test. For experiment 2 (urea concentration and LDH activity in the media), we performed a 2-way

Table 2. Genes of interest used in the quantitative reverse transcription PCR

Gene	Primer sequence (5' $\rightarrow$ 3'; F = forward, R = reverse)	Amplicon length (bp)	Accession no.	Reference
<i>ALB</i>	F GGGGTGTGTTTCGTCGAGAT R CTCACAGCAGTCAGCCATGT	293	NM_180992.2	Panda et al., 2015
<i>GAPDH</i>	F CAACATCAAGTGGGGTGATG R GGCATTGCTGACAATCTTGA	202	NM_001034034.2	Piechotta et al., 2013
<i>GHR1A</i>	F CCAGCCTCTGTTTCAGGAGTGT R TGCCACTGCCAAGGTCAAC	86	AY748827.1	Cummins et al., 2012
<i>GHR</i>	F ATGGCGGTATTGTGGATCAT R AGGATGTCGGCATGAATCTC	121	NM_176608.1	Cummins et al., 2012
<i>HNF4A</i>	F GAATCAACGGCGACATTCGG R AAGGCTGGGATGTACTTGCC	112	NM_001015557.1	—
<i>IGF1</i>	F ATGCCCCAAGCTCAGAAAG R GGTGGCATGTCTTCTTCACT	115	NM_001077828.1	Cummins et al., 2012
<i>IGFBP2</i>	F CACATCCCCAACTGTGACAA R GATCAGCTTCCCGGTGTTAG	114	NM_174555.1	Cummins et al., 2012
<i>NFKBIA</i>	F TGCAGGCCAACCACTACAAT R GACACCAGGTCGGGATTCTG	175	NM_001045868.1	—
<i>PCK1</i>	F CAACATCCGATTTCCGGGGT R TGTCAGCTCGATGCCAATCT	106	NM_174737.2	—
<i>PPIA</i>	F CACCGTGTCTTCTCGACAT R ACAGCTCAAAAGAGACGCGG	62	NM_178320.2	Green et al., 2010
<i>VIM</i>	F GTGCAGACTCTTACCTGCGA R CAGGCGGCCAATAGTGTCTT	129	NM_173969.3	—

Table 3. Cell count and cell viability of primary bovine hepatocytes obtained from adult cows¹

Variable	Cell count (vital cells/g of liver)			Cell viability (%)		
	Original protocol	Modified protocol	<i>P</i> -value	Original protocol	Modified protocol	<i>P</i> -value
Short warm ischemia	$2.7 \times 10^5 \pm 2.8 \times 10^5$	$4.7 \times 10^7 \pm 1.9 \times 10^7$	$P < 0.0001$	19.4 ± 7.1	79.1 ± 8.4	$P < 0.0001$
Long warm ischemia	$2.4 \times 10^5 \pm 7.6 \times 10^4$	$3.5 \times 10^6 \pm 1.5 \times 10^6$	NS	27.6 ± 5.9	37.1 ± 8.9	NS

¹Obtained from livers with short warm ischemia time (n = 12, 18 min) or long warm ischemia time (n = 12 livers, 28–30 min), perfused with the original protocol [n = 6 with short warm ischemia time and n = 6 with long warm ischemia time; Ehrhardt and Schmicke (2016)] or the modified protocol established for the present study (n = 6 with short warm ischemia time and n = 6 with long warm ischemia time). Values are given as mean $\pm$ SD. Significance values are for the comparison between the original and modified protocols.

ANOVA with the main effects of time, treatment (INS, DEX, INS+DEX, CTRL) and their interaction using a post hoc Tukey test. Overall, mRNA expression was also normally distributed; therefore, we performed a 2-way ANOVA with the main effects of day, treatment (INS, DEX, INS+DEX, CTRL) and their interaction. We used a Sidak test or Tukey test as a post hoc test, and a *P*-value of <0.05 was considered significant.

RESULTS

Experiment 1

The modified perfusion protocol ensured a higher cell count than that of the original protocol (Ehrhardt and Schmicke, 2016) when using livers with an SWI ($P < 0.0001$; Table 3). Both cell count and cell viability were higher in livers with an SWI than in those with an LWI when using the modified protocol ($P < 0.001$; Table 3).

Experiment 2

Based on the results from experiment 1, we performed experiment 2 using hepatocytes from livers with SWI isolated using the modified protocol.

Morphology. Figure 1 (a to d) shows primary bovine hepatocytes cultured in a sandwich culture at d 1 (24 h) with INS, DEX, INS+DEX, and CTRL. Morphologically, we detected no obvious difference in bovine hepatocytes with the different hormone substitutes in Williams' Medium E. Under all culture conditions, we detected single dead cells, indicated by a red nucleus and an unstained cytoplasm. The morphology of the hepatocytes was round. Cells attached as isolated, singular cells were from colonies consisting of 2 to 4 cells. When hepatocytes were cultured with DEX and INS+DEX, we observed bright elucidations between 2 hepatocytes, indicative of canaliculi formation (Figure 1, white arrows). At d 4 [Figure 1 (e to h)], the cellular morphology changed to cuboidal and polygonal, and hepatocytes formed cords reminiscent of hepatic trabeculae. We also detected bile canaliculi (bright

elucidations between hepatocytes; Figure 1, white arrows). In culture with INS (Figure 1e), the hepatocytes were somewhat flattened, whereas hepatocytes showed cytoplasmic protrusions (white stars) when cultured with DEX (Figure 1f), INS+DEX (Figure 1g), and even CTRL (Figure 1h). When hepatocytes were cultured with INS+DEX, the polygonal cell shape was dominant, with fewer obvious dead cells than in cultivation without DEX. With INS and CTRL, the red cells increased, but we did not assess those morphological changes quantitatively. Under all cultivation conditions, at d 4 we detected hepatocytes with 2 nuclei [Figure 1 (e to h), blue arrow]. The percentage of dead cells was higher in INS at d 4 than in DEX, INS+DEX, or CTRL (Table 4).

Urea Concentration and LDH Activity. We found that LDH activity increased at d 2 in all groups ($P < 0.0001$) and then decreased continuously, with the lowest LDH leakage detected at d 4. The LDH activity at d 2 was lower in media with INS and with DEX than in media with INS+DEX or CTRL ($P < 0.05$; Figure 2). Urea concentrations were low at d 1 and then increased continuously in media with DEX and INS+DEX ($P < 0.0001$). We found the highest urea production at d 4 for INS+DEX followed by DEX. In contrast, in media with INS and CTRL, we detected no comparable increase in urea (Figure 2).

mRNA Expression. We found that *HNF4A* and *ALB* were more highly expressed in hepatocytes cultured with INS and INS+DEX than in those cultured with DEX or CTRL. The substitution of DEX suppressed *VIM* and *NFKB1A* expression ($P < 0.0001$), but increased *PCK1* expression ($P < 0.0001$). The substitution of INS suppressed *PCK1* expression, but increased *HNF4A* and *ALB* expression ($P < 0.0001$; Figure 3).

The expression of *GHR*, *GHR1A*, and *IGF1* was suppressed by dexamethasone ($P < 0.0001$; DEX and INS+DEX), whereas INS distinctly increased *GHR*, *GHR1A*, and *IGF1* mRNA expression ($P < 0.0001$). The cultivation of primary bovine hepatocytes without any hormone substitutes (CTRL) also led to basic

Figure 1. Live/dead staining of primary bovine hepatocytes at (panels a–d) d 1 (24 h) or (panels e–h) d 4 (96 h) after collagenase perfusion in a sandwich culture system between 2 layers of collagen in Williams' Medium E supplemented with 100 nM insulin (INS), 100 nM dexamethasone (DEX), both (INS+DEX), or no hormone substitute (CTRL). Vital cells have a green cytoplasm (intracellular esterase activity, intact membrane), whereas dead cells appear with an unstained cytoplasm but a red-stained nucleus (ethidium-homodimer 1 binding to nucleic acids because of no intact membrane). Micrographs were taken with a phase-contrast microscope (Axiovert 200M with fluorescence; Zeiss, Oberkochen, Germany) and AxioVision software (<https://www.zeiss.com/microscopy/int/products/microscope-software/axiovision.html>). White arrows indicate a brightening between 2 hepatocytes indicative of canaliculi; blue arrows indicate hepatocytes with 2 nuclei; white stars indicate hepatocytes with cytoplasmic extensions (in one example, cells are additionally highlighted in violet); orange indicates cells in hepatocytic cords. Images on the left side are merged (phase contrast and fluorescence); images on the right side are phase-contrast only.

Figure 1 (Continued). Live/dead staining of primary bovine hepatocytes at (panels a–d) d 1 (24 h) or (panels e–h) d 4 (96 h) after collagenase perfusion in a sandwich culture system between 2 layers of collagen in Williams' Medium E supplemented with 100 nM insulin (INS), 100 nM dexamethasone (DEX), both (INS+DEX), or no hormone substitute (CTRL). Vital cells have a green cytoplasm (intracellular esterase activity, intact membrane), whereas dead cells appear with an unstained cytoplasm but a red-stained nucleus (ethidium-homodimer 1 binding to nucleic acids because of no intact membrane). Micrographs were taken with a phase-contrast microscope (Axiovert 200M with fluorescence; Zeiss, Oberkochen, Germany) and AxioVision software (<https://www.zeiss.com/microscopy/int/products/microscope-software/axiovision.html>). White arrows indicate a brightening between 2 hepatocytes indicative of canaliculi; blue arrows indicate hepatocytes with 2 nuclei; white stars indicate hepatocytes with cytoplasmic extensions (in one example, cells are additionally highlighted in violet); orange indicates cells in hepatocytic cords. Images on the left side are merged (phase contrast and fluorescence); images on the right side are phase-contrast only.

Table 4. Dead/live cells at d 2 and 4 in sandwich culture (mean $\pm$ SD)¹

Timepoint	Dead/live cells (%)			
	INS	DEX	INS+DEX	CTRL
Day 1	40.6 $\pm$ 10.0 ^a	31.5 $\pm$ 10.0 ^a	30.6 $\pm$ 3.6 ^a	40.0 $\pm$ 13.6 ^a
Day 4	67.0 $\pm$ 20.0 ^a	34.2 $\pm$ 11.7 ^b	37.0 $\pm$ 15.7 ^b	47.4 $\pm$ 22.3 ^b

^{a,b}Different superscript letters within a row indicate a significant ($P < 0.001$) difference between cell culture media additives.

¹INS = insulin; DEX = dexamethasone; CTRL = no hormonal substitute.

GHR and *GHR1A* mRNA expression, whereas expression was completely inhibited by DEX. The mRNA expression of *IGFBP2* increased with DEX but decreased completely with INS (Figure 3).

DISCUSSION

We previously published a protocol to gather hepatocytes from abattoir-derived liver, but we could not show any basal GHR expression (Ehrhardt and Schmicke, 2016). The aim of the present study was to establish an in vitro model for use in future studies to examine factors in adult bovine hepatocytes that affect the expression and signal transduction of GHR. With our original protocol, cell yield was relatively low (5.0×10^4 cells/g of liver) compared with, for example, hepatocytes gathered from fresh buffalo liver ($5.3 \pm 0.7 \times 10^7$ cells/g of liver (Panda et al., 2015)). Optimization of the protocol included 3 primary aspects: warm ischemia time, perfusion protocol, and substitution of insulin and dexamethasone in the culturing medium. The latter hormones are routinely added to primary hepatocyte media, but it is known from in vivo studies that insulin and dexamethasone clearly affect GHR expression (Godoy et al., 2013).

Warm ischemia time is a well-known critical parameter for the isolation of vital hepatocytes (Ikeda et al., 1992; Fisher et al., 1997; Hughes et al., 2006). According to the National Cancer Institute of the National Institutes of Health, warm ischemia time is defined “as the time a tissue, organ, or body part remains at body temperature after its blood supply has been reduced or cut off but before it is cooled or reconnected to a blood supply” (<https://www.cancer.gov/publications/dictionaries/cancer-terms/def/warm-ischemia-time>). With the cutoff of the blood supply, particularly at body temperature, cells are particularly vulnerable to damage due to hypoxia (Rauen and Groot, 2002). In particular, the warm ischemia time decreases cell viability (Caldwell-Kenkel et al., 1989, 1991; Ikeda et al., 1992). In the present study, we also demonstrated the negative effect of an LWI time on primary bovine hepatocyte viability. We further verified the findings

of Ehrhardt and Schmicke (2016), that gathering cells from abattoir-derived liver is possible, but that cell count and cell viability were higher in liver with a short rather than a long warm ischemia time. Therefore, for isolation of primary bovine hepatocytes liver with a short warm ischemia time should be used, as previously indicated by Panda et al. (2015).

Buffer composition, rewarming of the liver and the 2-step collagenase perfusion are other critical steps in gathering large numbers of vital primary hepatocytes. According to Johnson et al. (1996), the duration of collagenase digestion is important, one reason being that cell surface receptors can be destroyed by protease activity. In contrast to Ehrhardt and Schmicke (2016), who used 90 mg of collagenase P irrespective of liver weight, we used a concentration of 1 mg of collagenase per gram of liver tissue in the present study. With standardized collagenase digestion, which was previously successful for gathering human hepatocytes, the number of vital cells was improved (Lee et al., 2013). Moreover, we initiated some changes in the buffer composition and temperature. The increase in cold buffer volume used for rinsing the liver directly after withdrawal from the warm animal might also be important, as also noted for cows (Forsell et al., 1985) and horses (Bakala et al., 2003). The rewarming of the liver is necessary for the collagenase perfusion that requires body temperature (37–39°C) for optimal enzyme activity. In contrast to Ehrhardt and Schmicke (2016), in which rewarming occurred at 21°C, in the present study, the buffer was at 37°C and the duration of the perfusion steps was reduced. Toyokuni (1999) and Bhogal et al. (2010) note that during reperfusion, the production of reactive oxygen species leads to oxidative stress, a disturbance of the redox-homeostasis, resulting in damage to intracellular components (Zuo et al., 2015) and lipid oxidation that damages cell membranes (Halliwell 2006). In further studies, the addition of antioxidants such as ascorbic acid or tocopherol should be considered. A limitation of the present study was that we did not perform each modification step separately; we were unable to evaluate which modification step was most important.

Figure 2. Lactate dehydrogenase (LDH) activity (mU/mL) and urea concentrations (nmol/mL) measured in Williams' Medium E from adult primary bovine hepatocytes from d 1 to 4 after collecting the cells by using a collagenase perfusion in a sandwich culture system between 2 layers of rat tail collagen. CTRL = no hormone substitute; DEX = dexamethasone; INS = insulin; INS+DEX = insulin and dexamethasone. Values are given as medians with 25 and 95% quartiles. Error bars indicate minimum to maximum. Asterisks indicate significant differences at (*) $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

With liver obtained after SWI and gathering hepatocytes using the optimized protocol, in experiment 2 we tested in the influences of DEX, INS, both (INS+DEX), or no hormone additive (CTRL) on adult primary bovine hepatocytes. We detected differences in the morphology of cells, which was assessed using phase-contrast microscopy and live/dead fluorescence staining. In all cultivation conditions, the hepatocytes

developed an in-vivo-like polygonal cell shape, and the hepatocytes clustered into cords reminiscent of hepatic trabeculae, as previously described for rats (Tuschl und Mueller, 2006; Hewitt et al., 2007; Tchapanian et al., 2011). The collagen matrix below and above the hepatocytes in the sandwich culture promotes the formation of cell-to-cell contacts and supports an in vivo-like situation (Dunn et al., 1989). With the cells in contact

Figure 3. Relative mRNA expression of hepatocyte nuclear factor 4 α (*HNF4A*), nuclear factor- κ B-inhibitor α (*NFKBIA*), albumin (*ALB*), cytosolic phosphoenolpyruvate carboxykinase (*PCK1*), vimentin (*VIM*), growth hormone receptor total (*GHR*), *GHR1A*, *IGF1*, and IGF binding protein-2 (*IGFBP2*) in adult primary bovine hepatocytes in a sandwich culture system between 2 layers of collagen after culturing 6 and 72 h Williams' Medium E supplemented with insulin (INS), dexamethasone (DEX), both (INS+DEX), or no hormone substitute (CTRL). The mRNA data are given as $\Delta\Delta Ct$ (Ct = cycle threshold) relative to a calibrator (cells were collected immediately after the purification step and directly frozen at -80°C until analyses). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the reference gene. Values are given as medians with 25 and 95% quartiles. Error bars indicate minimum to maximum. Asterisks indicate significant differences at (*) $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

with an extracellular matrix at 2 sides, repolarization of the cells is supported, which leads to an in-vivo-like morphology and polarization of the hepatocytes (Berthiaume et al., 1996). One aspect of the polarization is that the hepatocytes form bile canaliculi with their apical membranes (LeCluyse et al., 1994), as determined by brightened and refracting structures between cells under phase-contrast microscopy, which in the present study, were detected at d 4. In future studies, the excretion potential of those bile canaliculi should be tested using fluorescence dyes. This repolarization of cells in a sandwich culture supports in vivo-like functions and prevents the hepatocytes from dedifferentiation. Both are crucial to support hepatocyte-like function (Talamini et al., 1997; Godoy et al., 2013; Zeigerer et al., 2017). As a marker of dedifferentiation in hepatocytes, the intermediary filament vimentin is widely used. The expression of vimentin is typical for mesenchymal but not for epithelial cells, so vimentin should be not expressed in hepatocytes (Godoy et al., 2009). In the present study, dexamethasone suppressed vimentin mRNA expression in adult primary bovine hepatocytes, and expression increased with the time of cultivation. These results confirm those of Godoy et al. (2010) with murine hepatocytes, in which dexamethasone also inhibited the expression of vimentin. In contrast, in the same study insulin showed the potential to increase the expression of vimentin, similar to the present study. Corresponding to this finding, the percentage of dead/live cells was higher at d 4 with INS compared with DEX, INS+DEX, and CTRL.

To evaluate the hepatocyte-like function, we measured LDH activity and urea concentration in the media. Leakage of the cytoplasmic enzyme LDH can be used as an indirect marker of hepatocyte viability (Mitchell et al., 1980). The results of the present study indicated high LDH leakage at the beginning of the culture, decreasing continuously thereafter, which might indicate an increase in the rate of cell death immediately after cell separation and stabilization. It has to be noted that in the sandwich culture, no useful standardization with relation to cell count was possible. But the single wells were morphologically assessed each day to determine that the cell count was always comparable between wells, with one exception. At d 4 more dead cells were detected in INS cultivated cells compared with DEX, INS+DEX, and CTRL. Therefore, the lower urea production on d 4 might also have been because fewer cells were vital. Sidhu et al. (2004) indicated a suppressive effect of dexamethasone in the medium on LDH leakage in rat hepatocytes, which we could not confirm in the present study. On d 4 of the present study, we detected no effect of either INS or DEX. However, the highest urea production was found on d

4 with INS+DEX, and even DEX alone had a stimulatory effect on urea production. In agreement with this Okun et al. (2015), also found an increased production of urea by dexamethasone, and Nebes and Morris (1988) found that dexamethasone has a direct effect on carbamoylphosphate synthetase I, argininosuccinate synthase, argininosuccinate lyase, and arginase 1, with increases in mRNA expression of urea in culture with dexamethasone.

The underlying reason for dexamethasone addition to the medium for hepatocyte culture is not only to depress possible dedifferentiation, but also for its anti-inflammatory potential, which was indicated in the present study by the suppression of I κ B α expression. We used I κ B α to assess the inflammatory status of the hepatocytes, because it is an indirect measure of nuclear factor- κ B activation (Bottero et al., 2003; Paciolla et al., 2011). As well, HNF4 α is used as a marker for hepatocyte-like differentiation (Panda et al., 2015; Gleich et al., 2016). In contrast to the present study, Onica et al. (2008) and Oyadomari et al. (2000) reported a stimulation of HNF4 α expression in human and rat hepatocytes, which might have been due to species differences.

To summarize, these data showed that dexamethasone prevented hepatocytes from dedifferentiation and apoptosis/inflammation, but we also detected hepatocyte-like functions in the production of albumin and the downregulation of PEPCK-C (*PCK1*) expression after insulin administration. Therefore, short-term cultivation without dexamethasone but with an alternative anti-inflammatory drug might be necessary, because we clearly demonstrated that the addition of dexamethasone distinctly downregulated expression of *GHR1A* and *GHR*. In contrast, the addition of insulin clearly increased expression of *GHR*, *GHR1A*, and *IGF1* mRNA, consistent with in vivo studies in which insulin increases *GHR1A* and *IGF1* expression (Butler et al., 2003; Rhoads et al., 2004). As well, the suppression of *GHR* after 5 d of dexamethasone application in cows (Maciel et al., 2001) was consistent with the in vitro results of the present study. In primary rat hepatocytes, a high dose of dexamethasone decreased the expression of *GHR* mRNA (Beauloye et al., 1999). In the present study, 100 nM insulin was added to the medium, in contrast to Ehrhardt and Schmicke (2016), in which only 1.74 nM insulin was added to the medium. Therefore, the low insulin and high dexamethasone concentrations in the cell culture medium were responsible for the low *GHR* expression in the previous study (Ehrhardt and Schmicke, 2016). However, with 100 nM insulin, additional hepatocytes showed fibroblastic protrusions, and the key enzyme for gluconeogenesis, PEPCK-C, was strongly downregulated. In further studies, an optimal

(and physiological) insulin concentration in the medium should be elucidated to support GHR expression with increased physiological insulin concentrations. Moreover, other anti-inflammatory agents should be chosen. Dexamethasone at 100 nM is routinely added to hepatocyte medium (Godoy et al., 2013). We used this concentration in the present study and in that of Ehrhardt and Schmicke (2016), but we clearly showed that this dose suppressed GHR expression. Additional detection of protein levels by detecting GHR protein with Western blot analysis would further confirm our findings but was not performed in the present study. To further characterize the functional GHR-specific signal transduction pathway, analyses should be initiated in future studies.

We showed an increase in *IGFBP2* mRNA expression with dexamethasone, but insulin blocked the increase. This strong inhibition of *IGFBP2* by insulin is also shown in vivo in lactating dairy cows at 73% (McGuire et al., 1995). In primary rat hepatocytes, insulin also inhibited *IGFBP2* expression (Böni-Schnetzler et al., 1990; Scharf et al., 1996). When 100 nM insulin was added to the medium, comparable to the dose used in the present study, no IGFBP-2 was detected in the medium (Scharf et al., 1996).

Based on our findings, the influence of warm ischemia time should not be underestimated; a short time between slaughter and cooling of the liver tissue had a positive effect on cell count and cell viability. Distinct improvements to the original isolation protocol (Ehrhardt and Schmicke, 2016) led to a massive amplification in the amount of viable adult primary bovine hepatocytes. Cultivation without dexamethasone ensured a basal GHR expression. We also observed in vivo results by demonstrating the increased expression of GHR and decreased expression of IGFBP-2 with insulin. This finding also indicated that GHR expression is reactive, revealing primary bovine hepatocytes in sandwich culture as a potential in vitro model for further functional studies on GHR expression.

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